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# Short-term CO<sub>2</sub> exposure and temperature rise effects on metazoan meiofauna and free-living nematodes in sandy and muddy sediments: Results from a flume experiment

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## ABSTRACT

Global concern over increasing CO<sub>2</sub> emissions, and the resultant CO<sub>2</sub> driven temperature rises and changes in seawater chemistry, necessitates the advancement of understanding into how these changes will affect marine life now and in the future. Here we report on an experimental investigation into the effects of increased CO<sub>2</sub> concentration and elevated temperature on sedimentary meiofaunal communities. Cohesive (muddy) and non-cohesive (sandy) sediments were collected from the Eden Estuary in St. Andrews, Scotland, UK, placed within a flume setup and exposed to 2 levels of CO<sub>2</sub> concentration (380 and 750 ppmv, current at the time of the experiment, and predicted CO<sub>2</sub> concentration by 2100, respectively) and 2 temperature levels (12 °C and 16 °C, current in-situ and predicted temperature by 2100, respectively). We investigated the metazoan meiofauna and nematode communities before and after 28 days of exposure under these experimental conditions. The most determinative factor for abundance, diversity and community structure of meiofauna and nematodes was sediment type: on all levels, communities were significantly different between sand and mud sediments which agrees with what is generally known about the influence of sediment structure on meiofaunal organisms. Few CO<sub>2</sub> and temperature effects were observed, suggesting that meiofauna and nematodes are generally much less responsive than, for instance, microbial communities and macrofauna to these environmental changes in estuarine environments, where organisms are naturally exposed to a fluctuating environment. This was corroborated by the observed effects related to the different seasons in which the samples were taken from the field to run the experiment. After 28 days, meiofauna and nematode communities in muddy sediments showed a greater response to increased CO<sub>2</sub> concentration and temperature rise than in sandy sediments. However, further study is needed to investigate the underlying mechanisms and meiofauna species-specific resilience and responses to ocean acidification and warming, and their interactions with other biota, to understand what such changes may mean for meiofauna communities and the ecosystem processes and functions they contribute to.

## 1. Introduction

In the past 800 k years CO<sub>2</sub> concentrations in the atmosphere have remained in the range of 172–300 ppm by volume (ppmv) (Luthi et al.,

2008). Since the start of the industrial era atmospheric CO<sub>2</sub> concentrations have increased dramatically and are currently exceeding 400 ppmv (parts per million in volume), with predictions of between 550 and 900 ppmv by 2100 (Cao and Caldeira, 2008; Hoegh-Guldberg

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and Bruno, 2010; IPCC, 2014). Of all the anthropogenic CO<sub>2</sub> emitted into the atmosphere, about 30% has been absorbed in the surface ocean (< 200 m) so far (Sabine et al., 2004). As this CO<sub>2</sub> is absorbed, it changes the seawater carbon chemistry and reduces pH in a process that is called “ocean acidification” (Gattuso and Hansson, 2011). Ocean acidification is already occurring (e.g. Caldeira and Wickett, 2003) and is predicted to worsen in the near and distant future with a reduction from pre-industrial pH levels of 8.2 to a projected global average of 7.8 in 2100 (Branch et al., 2013; IPCC, 2014). Ocean acidification, however, is a spatially variable phenomenon that is strongly modified by local conditions, particularly in coastal areas, which could lead to local ocean acidification hot spots. The accumulation of CO<sub>2</sub> in the atmosphere also increases the natural greenhouse effect and continues to drive global warming (IPCC, 2014). As a result, each of the last three decades has been successively warmer at the Earth's surface than any preceding decade since 1850, and whilst surface temperature increases of up to 4.8 °C are predicted by 2081–2100 (IPCC, 2014), the temperature in the top 100 m of the ocean is expected to increase by 0.6 to 2.0 °C by 2100 (Collins et al., 2013). A variety of biological responses to ocean acidification and warming have been measured across a range of taxa, with substantial negative effects on individual responses (such as survival, calcification, growth and reproduction), ecological interactions (such as trophic relationships and organism behaviour) and community characteristics (such as abundance, diversity, production and biomass) (Alsterberg et al., 2013; Danovaro et al., 2004; Gingold et al., 2013; Kroeker et al., 2010). However, significant variation in the sensitivity of marine organisms is observed with for instance calcifying organisms being generally more susceptible to pH reductions than non-calcifying organisms, and pH reductions and temperature increases having different effects depending on the developmental stage or even sex (Ellis et al., 2017) of the organisms being studied and the gradients and shifts of complex ecological interactions (Ingels et al., 2012; Kroeker et al., 2010).

Marine organisms will be faced with a wide range of stressors in our future oceans, and it is therefore imperative that experimental approaches include multiple stressors in their designs to assess species, community, and ecosystem-level responses. Biological responses to ocean acidification and warming (OAW) will depend on physiological trade-offs or energy allocation to sustain the performance, survival and fitness of organisms (Brown et al., 2004; Findlay et al., 2011; Pörtner, 2008; Queirós et al., 2015). However, information from studies focusing on particular species or life-stages of certain species is - although crucial in documenting autecological processes and responses - insufficient to predict future change on the level of ecosystems considering the wide range of trophic and non-trophic interactions between species (Russell et al., 2011). Therefore, approaches that focus on groups of marine organisms that have ecological and functional significance are needed to document the effects and responses to OAW at a community level (Riebesell et al., 2010). In addition to the requirement for more studies integrated over various levels of biological organisation (Ingels et al., 2012), there is also an urgent need for more studies that cover the additive and synergistic effects of ocean acidification and warming occurring simultaneously (Pörtner, 2008).

The meiofauna comprises the small-sized organisms (generally between 32 and 63 µm and 1 mm; the lower size limit varies in the literature) in the benthos, whose morphology, physiology and life history characteristics have evolved to exploit the interstitial sedimentary space. They occur in often high abundance in sediments worldwide, and are phylogenetically very diverse (Balsamo et al., 2012). The most abundant metazoan meiofaunal organisms in marine sediments are consistently the nematodes and copepods, with nematodes having colonized virtually every moist habitat that can sustain metazoan life. Meiofauna are important contributors to the physical, chemical and bi-

ological properties of sediments, the resilience of those sediments, and their role in benthic food webs has been amply documented (Schratzberger and Ingels, 2017). We can therefore consider them as an important ecological component of benthic ecosystems and alterations to their communities as a consequence of OAW may give much needed insights in to how benthic ecosystem structure and function may respond in future oceans.

Metazoan meiofauna generally comprise non-calcifying, infaunal invertebrates with low mobility. They are naturally exposed to large fluctuations of pore water pH and CO<sub>2</sub> concentrations and are therefore considered likely to be more tolerant than other animal groups to higher CO<sub>2</sub> concentrations (Gattuso and Hansson, 2011; Widdicombe et al., 2011) and temperature (Giere, 2009; Moens et al., 2013), particularly in shallow-water subtidal and intertidal coastal environments (Giere, 2009). This (assumed) tolerance, in addition to increased logistic effort associated with multiple stressors and necessary replication, as well as the required meiofaunal taxonomic expertise, may explain why relatively few studies so far have documented the effects of OAW on metazoan meiofauna (Hale et al., 2011; Meadows et al., 2015; Sarmiento et al., 2016). That being said, there are several studies that have investigated OA or increased CO<sub>2</sub> concentration as a single stressor on metazoan meiofauna or nematodes (Barry et al., 2004; Carman et al., 2004; Dashfield et al., 2008; Ishida et al., 2013; Ishida et al., 2005; Kurihara et al., 2007; Takeuchi et al., 1997; Thistle et al., 2005; Widdicombe et al., 2009; Widdicombe et al., 2011). Some of these studies, however, have focused on effects associated with injected CO<sub>2</sub> or simulated CO<sub>2</sub> leakage or release in the context of Carbon dioxide Capture and Storage (Barry et al., 2004; Carman et al., 2004; Schade et al., 2016; Thistle et al., 2005) rather than ocean acidification in a climate change context. Effects of rising temperatures on metazoan meiofauna and nematodes in the context of climate change have equally been covered in some detail in existing literature (e.g. Gingold et al., 2013; Ingels et al., 2012). The OAW effects reported in these studies vary, depending on whether species or communities were studied, and which ontogenic stage was considered, and of course whether single stressors or multiple stressors were applied. Notably the influence of ecological interactions such as those between the macrofauna and meiofauna (trophic and competitive interactions), but also between individual species, on stressor responses creates a complex view of community dynamics in response to OAW and requires further study.

In the present study we report on the responses of meiofauna and nematode communities to OAW in a flume experiment where we exposed two types of intertidal sediment communities (muddy and sandy) to increased temperatures (+ 4 °C) and CO<sub>2</sub> concentrations (750 ppmv) in a fully crossed design. We sieved the sediments to exclude macrofauna, and hence were able to test more direct OAW effects on meiofauna in the absence of macro-meiofauna interactions. We used intertidal sediments, in which meiofauna organisms experience temperature and pH variations, amongst others, over tidal and diurnal cycles and whilst they may be well adapted to cope with such fluctuations, they may also be already pushed to the physiological edge of their tolerances. The main aim of our study was therefore to assess whether OAW has an effect on meiofauna and nematodes on a community level in intertidal sandy versus muddy sediments. Little is understood on how infaunal responses to OAW may differ between different sediment types despite the acknowledgement that benthic communities in different sediment types are distinct from each other. In addition, recent reviews document meiofauna and nematode responses to OAW and that the meiofauna (in particular foraminifera, nematodes, copepods and ostracods) are useful in detecting and monitoring environmental change and anthropogenic impacts (Zeppilli et al., 2015). Scientific endeavours to assess responses of marine ecosystems to naturally occurring and anthropogenically induced stressors are currently hampered by a lack of 1) understanding of which taxa will be affected in

marine communities and their importance for ecosystem functioning, 2) multistressor experiments which can indicate complex changes and biological interactions on a community and ecosystem scale, and 3) understanding how different marine habitats respond differently to these stressors. Addressing these three issues will improve our ability to accurately assess climate change effects and help predict ecosystem shifts across entire systems (Queirós et al., 2015; Russell et al., 2011; Zeppilli et al., 2015). It is in this context that we addressed the following questions with regards to meiofauna responses to OAW: 1) Does OAW affect meiofauna and nematode communities in shallow-water sediments?; 2) If there are OAW effects, do these effects differ between sandy and muddy shallow-water sedimentary environments?; 3) If there are OAW effects, does warming and ocean acidification together affect meiofauna and nematode communities more strongly than either stressor separately? The hypotheses associated with these questions are H1: “OAW affect meiofauna and nematode communities in terms of abundance, diversity and evenness”; H2: “Meiofauna and nematode community responses to OAW are different in muddy versus sandy sediments”; and H3: “ocean acidification and warming together affect the meiofauna and nematode communities more strongly than either stressor in isolation”.

## 2. Material and methods

### 2.1. Experimental setup

#### 2.1.1. Sediment collection

Two different sediment types were collected from the Eden Estuary near St Andrews, Fife, Scotland over four campaigns (October 2011; April 2012; June 2012 and July 2012). Cohesive surface sediment ( $< 63 \mu\text{m}$ ) was collected from intertidal mudflats ( $56^\circ 21.9 \text{ N}$ ,  $2^\circ 50.883 \text{ W}$ ), and permeable sediment was collected from the West Sands bank near the mouth of the estuary ( $56^\circ 22 \text{ N}$ ,  $2^\circ 49 \text{ W}$ ). Surface sediment (the top oxic layer as visually determined by the sediment colour change of the suboxic layer) was collected in the field, by hand, to a depth of no  $> 2 \text{ cm}$ . This sediment was placed directly into food-grade buckets and returned to the laboratory for sieving. All sediment was sieved ( $500 \mu\text{m}$  for cohesive;  $1 \text{ mm}$  for permeable) in a seawater bath (UV sterilised,  $10 \mu\text{m}$  filtered, salinity  $\sim 35$ ) to remove macrofauna and larger shells and stones, and was left to settle for 48 h prior to removal of the supernatant. This allowed the finer particles of the sediment, along with the meiofauna, to settle. Each sediment type was homogenised and added to custom-built flume tanks, to a depth of  $10 \text{ cm}$

(Fig. 1). Seawater ( $1 \mu\text{m}$  filtered, UV treated, and salinity maintained at 35 through a brine tank set up) was carefully added to each tank and left for a further 48 h before it was replaced with new seawater (UV treated,  $1 \mu\text{m}$  filtered,  $\sim 35 \text{ psu}$ ). Each tank was then bubbled with ambient air ( $380 \text{ ppmv}$ ) for 72 h prior to implementation of the experimental  $\text{CO}_2$  and temperature regimes.

#### 2.1.2. Flume tanks and environmental regimes

Each sediment type filled three flume tanks (L  $120 \text{ cm} \times \text{W}$   $30 \text{ cm} \times \text{H}$   $30 \text{ cm}$ ; approx.  $3.24 \times 10^4 \text{ cm}^3$  volume and approx.  $10 \text{ cm}$  height) per campaign ( $n = 4$ ), with a continuous, recirculating and unidirectional flow of overlying water over the sediment surface (PISCES SC50 water pump, flow rate  $\sim 6 \text{ cm/s}^{-1}$ ) for the duration of the experiment (28 days). All six flume tanks in each campaign were subjected to a 12-h light/dark cycle (Osram daylight tubing L3677, T8, 36 watts,  $1200 \text{ mm}$  long; two per tank). Two temperature ( $12^\circ \text{C}$ ,  $16^\circ \text{C}$ ) and two  $\text{CO}_2$  regimes ( $380 \text{ ppmv}$ ,  $750 \text{ ppmv}$ ) were used in a fully-crossed factorial design to provide 3 replicate flume tanks per unique treatment ( $n = 4$ ), with replication spread temporally over campaigns (Fig. 1).  $\text{CO}_2$  was bubbled into the water column to reach equilibrium and concentrations in each tank were monitored using a Li-Cor 820  $\text{CO}_2$  gas analyser (Biogeosciences). Each tank was also individually aerated to achieve oxygen saturation. Temperature was controlled by submerged titanium heaters with a digital regulating unit (Aqua Medic T-meter) for the duration of the experiment.

#### 2.1.3. Carbonate system analysis

Water samples were taken weekly in light and dark conditions for total alkalinity (TA,  $30 \text{ ml}$ ) and DIC ( $12 \text{ ml}$ ) and poisoned with  $50 \mu\text{l}$  saturated  $\text{HgCl}_2$  solution. Samples were stored in acid-washed, rinsed, capped glass bottles and refrigerated ( $4^\circ \text{C}$ ) prior to analysis. TA samples were analyzed using an automatic potentiometric 196 titrator (888 Titrando, Metrohm, Switzerland) with Tiamo® V 2.1 software. A three-point calibration was performed using buffer solutions pH 4, 7 and 9 (Metrohm UK Ltd.) prior to analysis. The precise volume of HCl acid added was plotted against pH, and this curve was then logged to produce a straight line. The gradient of this line was used to calculate TA (Dickson et al., 2007). Certified  $\text{CO}_2$  reference material (Andrew G. Dickson, Scripps Institution of Oceanography, California, USA) was used to monitor the sampling accuracy of the titrator (Dickson et al., 2003). DIC was determined using a CM140 Total Inorganic Carbon Analyser (UIC Inc., USA) following Dickson et al. (2007). Blanks were run at the start of each analysis to calibrate the machine and to deter-

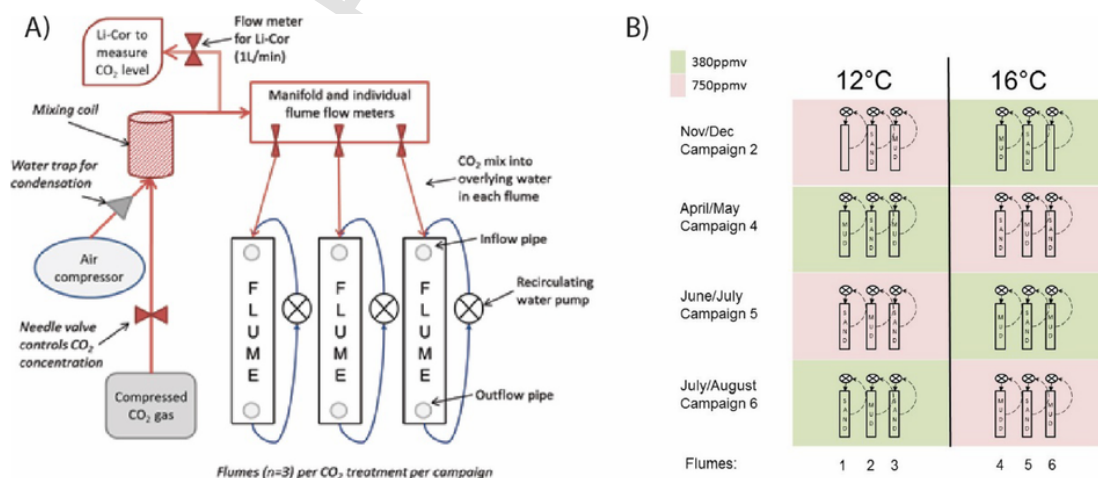


Fig. 1. (A) Schematic diagram of experimental setup and circulation system; (B) schematic of the experimental treatments in the flume tanks (empty flumes in Campaign 2 were not used for this study).

mine the carrier gas carbon content. Seawater standards of known concentration were then also run through the DIC machine to ascertain precision and accuracy within  $\pm 0.01 \text{ mmol l}^{-1}$ . Prior to each analysis a standard solution of sodium bicarbonate ( $\text{NaHCO}_3$ ), made to known concentrations, was run until a precision of 0.03% deviation was achieved from three consecutive samples. IAPSO seawater samples (commercially available) were also routinely run through the machine to check accuracy.

#### 2.1.4. Nutrient analysis

Water samples were collected weekly from each flume using 50 ml syringes and a  $0.45 \mu\text{m}$  filter, and were stored in clean 45 ml centrifuge tubes for freezing at  $-20^\circ\text{C}$ . Samples were defrosted prior to analysis, and gently mixed through manual inversion to reduce saline stratification in the sample tubes. An auto-analyser (LaChat 8500 Flow Injection) analyzed four nutrients from each sample in triplicate: ammonium ( $\text{NH}_4$ ), phosphate ( $\text{PO}_4$ ), nitrite + nitrate ( $\text{NO}_2 + \text{NO}_3$ ) and Silicate (Si). Low nutrient concentration seawater (salinity 35) was used for standard preparation and machine calibration.

#### 2.2. Sampling

Sediment samples were taken towards the middle of the flume using  $4 \times 10 \text{ ml}$  syringes (1.4 cm diameter; 6–7 cm deep) at T0 and T28 for each flume and campaign. Syringes were frozen at  $-20^\circ\text{C}$  to allow for different types of analyses. In the laboratory, the sediments of the four syringes per sampling point were pooled ( $6.158 \text{ cm}^2$  surface area) and left to thaw in 4% formaldehyde in order to avoid degradation of the meiofauna during thawing. Pooling of the four syringes was conducted to remove meiofaunal spatial heterogeneity in the flume sediments. The pooled samples were then washed through a 1 mm sieve onto a  $63 \mu\text{m}$  sieve. To extract the meiofauna from the sediment fraction, the material that remained on the  $63 \mu\text{m}$  sieve was thoroughly mixed using a plastic paddle with Ludox TM-50 (specific gravity 1.15) in 500 ml glass beakers and left for 40 min to enable density separation to occur. This process was repeated 3 times (Somerfield and Warwick, 1996) whereby each time the supernatant Ludox containing the meiofauna organisms was decanted and washed. The final washed and extracted sample was then stored in 75% Industrial Methylated Spirit until further analysis. From each sample, a subsample of between 20% or 30% of total sample volume was taken and meiofauna major taxa were counted under stereoscopic microscope using (Higgins and Thiel, 1988). All Nematodes (or 100 if subsample contained more) were picked out and mounted on glass slides for genus identification under compound microscope (Somerfield and Warwick, 1996) using appropriate reference materials (Platt and Warwick, 1983, 1988; Warwick et al., 1998). Whilst the protozoan group Sarcostigophora were counted (they include Foraminifera, flagellates and radiolarians) we have limited our results and discussion to the metazoan meiofauna because they are ecologically and biologically very different to protozoans and we have not identified the different taxa within the Sarcostigophora. The use of the term meiofauna in the rest of the study refers to metazoans only. A total of 11,441 meiofauna (6146 nematodes) individuals were identified for this study.

#### 2.3. Data processing and statistical analysis

Density and diversity: Meiofauna abundance values were calculated as total sample abundance converted to number of individuals per  $10 \text{ cm}^2$ . Diversity was calculated as number of major taxa (meiofauna) and number of genera (nematodes), Shannon-Wiener's diversity index and Pielou's evenness index (using PRIMER v7, Clarke and Gorley, 2015). Graphs and non-metric MDS plots (Clarke and Gorley, 2015)

were used to visualize sampling and experimental results. Univariate tests for differences (density, number of taxa, Shannon diversity and Pielou's evenness) were conducted with non-parametric analyses of variance (PERMANOVA) using Primer v7 (Clarke and Gorley, 2015) and the add-on PERMANOVA + (Anderson et al., 2008). Because of the complex design of the experimental setup we applied a 4-way PERMANOVA test (fixed Factors [levels]: Temperature [ $12^\circ\text{C}$ ,  $16^\circ\text{C}$ ],  $\text{CO}_2$  concentration [ $380$ ,  $750 \text{ ppmv}$ ], Time [day 0, day 28], Sediment [sand, mud]) on the meiofauna and nematode data, followed by 3-way PERMANOVA tests on split sediment-type data (i.e. sand, mud; fixed Factors [levels]: Temperature [ $12^\circ\text{C}$ ,  $16^\circ\text{C}$ ],  $\text{CO}_2$  concentration [ $380$ ,  $750 \text{ ppmv}$ ], Time [day 0, day 28]) and 2-way PERMANOVA tests on split sediment type-time data sets (Sand-T0, Sand-T28, Mud-T0, Mud-T28; fixed Factors [levels]: Temperature [ $12^\circ\text{C}$ ,  $16^\circ\text{C}$ ],  $\text{CO}_2$  concentration [ $380$ ,  $750 \text{ ppmv}$ ]). In the few cases where the number of permutations was  $< 100$  we used Monte Carlo values, and the Estimated Components of Variation were sometimes used to interpret the size of the effects.

Communities: The multivariate meiofauna and genus matrices were also subjected to multivariate statistics using Primer v7 (Clarke and Gorley, 2015) and the add-on PERMANOVA + (Anderson et al., 2008). Here we also applied a 4-way PERMANOVA test (Factors [levels]: Temperature [ $12^\circ\text{C}$ ,  $16^\circ\text{C}$ ],  $\text{CO}_2$  concentration [ $380$ ,  $750 \text{ ppmv}$ ], Time [day 0, day 28], Sediment [sand, mud]) on the meiofauna and nematode community data, followed by 3-way and 2-way tests on split data as done for the abundance and diversity data, with factors and levels as identified above. The meiofauna and nematode community data were standardised and transformed prior to analysis (meiofauna: 4th root, nematodes: sq. root) to account for sample size differences (20% vs. 30%) and downweight the influence of numerically dominant major taxa/nematode genera. Bray-Curtis similarity was used for both meiofauna and nematode community data. Significance was assessed as  $p < 0.05$ . Non-metric MDS plots were created to accompany the non-parametric tests. Cluster analyses (including SIMPROF test at 5% significance) were performed to analyse significant groupings of samples which were then superimposed on nMDS outputs.

The PERMANOVA tests were used to assess treatment differences as well as assessing the nature of the differences of each factor and their potential interactions. These analyses were followed by pairwise comparisons where significant differences occurred. PERMDISP analyses were performed where appropriate to identify whether significant PERMANOVA results were caused by differences in location in Bray-Curtis (multivariate) or Euclidean (univariate) space or the homogeneity of dispersion of the samples within group, or a combination of both (Anderson et al., 2008). Differences in the multivariate dispersion of assemblage data may indicate stress in the observed communities, and can contribute to our understanding of how communities react to temperature and  $\text{CO}_2$  concentration increases in our case (Anderson et al., 2008). Although increased variability may be an artefact caused by low abundance in samples (causing the resemblance measure to vary to a much greater extent compared to high abundance samples), the application of standardisation (transforming absolute abundance into relative abundance) renders the test more useful for assessing stress in communities, although caution with interpreting the results is recommended (Anderson et al., 2008).

The potential effects of the different times at which sampling for the experiment occurred and the six different flumes that were used were assessed by means of 2-way PERMANOVAs, and accompanying pairwise tests where necessary (Factors [levels]: Campaign, Ca [2, 4, 5, 6]; Flume, Fl [1, 2, 3, 4, 5, 6]). These 2-way tests were performed on meiofauna abundance, diversity and community data and nematode diversity and community data, and were repeated for the full data (sand and mud together), sand and mud separately, and each of the Sand-T0, Sand-T28, Mud-T0, Mud-T28 data sets. In some cases, and because of

the design, there was no replication between crossed factor levels, causing the exclusion of interaction terms in the PERMANOVA or even rendering tests invalid; this has been indicated in the test result tables and its implications have been considered in the interpretations of the results.

### 3. Results

#### 3.1. Environmental variables

The pH level was  $7.90 \pm 0.022$  (standard error) and  $8.03 \pm 0.024$  for the control treatments (380 ppmv), 12 °C and 16 °C, respectively. The pH level for the high-CO<sub>2</sub> treatments was  $7.86 \pm 0.023$  and  $7.77 \pm 0.031$  for 12 and 16 °C, respectively. Total alkalinity (TA) and DIC were slightly higher in the muddy sediments (TA = 2.8 mmol/kg, DIC 2.6–2.7 mmol/kg) than in the permeable sandy sediments (TA = 2.6–2.7 mmol/kg, DIC 2.3–2.5 mmol/kg) throughout the experiment. This is due to the fact that there are higher benthic respiration rates in the muddy flumes compared to the sandy flumes, which in turn would have stimulated CaCO<sub>3</sub> dissolution (release of CO<sub>2</sub> from respiration increase the CaCO<sub>3</sub> dissolution). The CaCO<sub>3</sub> dissolution generated both DIC and TA; Nutrients showed overall higher concentrations in the muddy sediment compared to the sandy sediment (except for phosphate). Nutrient, DIC and TA data are presented in Table 1. We observed that DIC levels were highest in elevated CO<sub>2</sub> treatments (750 ppmv), which was expected due to the addition of CO<sub>2</sub> compared to the 380 ppmv treatments. This was not reflected in TA, as CO<sub>2</sub> invasion only affects DIC and not TA.

#### 3.2. Meiofauna abundance, diversity and community structure

##### 3.2.1. Meiofauna abundance

Meiofauna abundance was highly variable with a minimum of 75.8 and maximum of 9841.6 ind. 10 cm<sup>-2</sup> and averaging  $1735.3 \pm 2136.5$  ind. 10 cm<sup>-2</sup>. A 4-way PERMANOVA test on meiofauna abundance indicated only significant sediment-type differences ( $p = 0.001$ ) caused by a combination of differences in dispersion (greater abundance variability in the muddy sediments, PERMDISP,  $p = 0.007$ ) and actual abundance differences (greater abundance in muddy sediments, Fig. 2; Table S1). Sandy sediments contained on average  $374.4 \pm 586.8$  ind. 10 cm<sup>-2</sup> whilst muddy sediments contained  $3096.1 \pm 2263.2$  ind. 10 cm<sup>-2</sup>. Despite an average decrease in abundance between day 0 and day 28 for both sandy and muddy sediments (Fig. 2A), high variability in recorded values rendered the time effect insignificant ( $p = 0.109$ ). Further evidence for this is provided by the PERMDISP

analysis between sediment-time groups ( $p = 0.001$ ), with the high abundance variability in mud and sand sediments at day 0 and day 28 causing an increased dispersion effect. As average abundance decreases throughout the duration of the experiment for both sediment types we also see a decrease in the variability of meiofauna. The only treatment effect observed was for CO<sub>2</sub> exposure in the mud samples after day 28 (Table S1, Fig. 2B), with significantly lower abundance in the 750 ppmv samples compared to the 380 ppmv samples. This density decrease was caused by a reduction across several taxa, with major decreases in numbers of nematodes, copepods, and ostracods (Fig. 2C–D).

##### 3.2.2. Meiofauna diversity

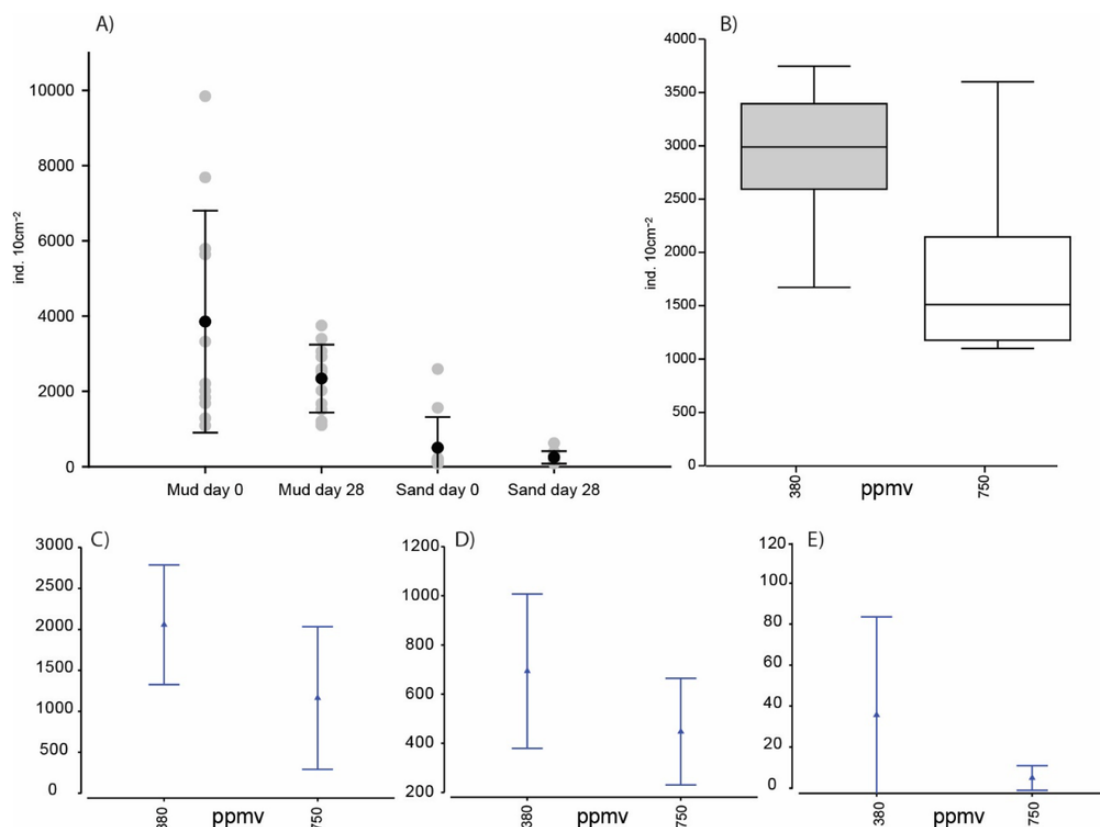
In terms of diversity, a wider range of effects was observed. The number of major meiofauna taxa or taxon richness (S) ranged between 1 and 8, averaging 3.5 across all samples, and samples were dominated by Nematoda (84.4%) and Copepoda (11.3%), whilst Sarcomastigophora and Oligochaeta each represented just over 1%, and Acari, Bivalvia, Gastrotricha, Kinorhyncha, Ostracoda, Rotifera and Turbellaria were each present in much lower abundance (< 1%). Taxon richness was significantly higher in muddy compared to sandy sediments ( $p = 0.001$ , Fig. 3) with no dispersion differences (PERMDISP,  $p = 0.749$ ); sandy sediments contained between 1 and 7 taxa, averaging  $1.8 \pm 1.4$  taxa per sample, whilst muddy sediments contained between 3 and 8 taxa, averaging  $5.3 \pm 1.8$  taxa per sample. A number of 2-factor interactions were significant (Ti x Se, Ti x CO<sub>2</sub>, Se x Te). Pairwise testing for these interactions and further investigation using 3-way PERMANOVA tests (Ti, Te, CO<sub>2</sub>) on sandy and muddy sediment sample groups separately revealed: [1] a time effect (day 0 vs. day 28) in sandy but not in muddy sediments; [2] a severe sediment effect at day 0 as well as day 28; [3] some evidence for a time effect in the CO<sub>2</sub> control (380 ppmv) and enriched (750 ppmv) samples (borderline  $p$ -values of 0.051, and 0.081, respectively); [4] a difference between day 0 samples in the 380 ppmv and 750 ppmv treatment ( $p = 0.037$ ); [5] a clear sediment effect in both 12 °C and 16 °C treatments ( $p = 0.001$ ), and [6] a temperature effect in muddy sediments at day 0 ( $p = 0.014$ ). Taking all these test results together (including the accompanying PERMDISP results), they suggest that [1] there are substantial differences between starting communities mostly owing to sediment differences and variability of taxon presence, [2] sediment differences may obscure other treatment effects, and [3] there is some effect due to the time spent in the flume setup (Table S1). Observations [1] and [2] were confirmed by a significant temperature effect on diversity at day 0 ( $p = 0.025$ ) and not day 28 ( $p = 0.3$ ) in muddy sediments despite the fact that there had not been substantial exposure to higher temperatures yet at day 0 (pairwise comparisons in 3-way PERMANOVA (Ti, Te, CO<sub>2</sub>) in muddy sediments), and the significant differ-

Table 1

Mean seawater chemistry values for the different treatments. Values in italics denote standard errors for means.

	Muddy sediment				Sandy sediment			
	12 °C	16 °C	12 °C	16 °C	12 °C	16 °C	12 °C	16 °C
Temperature	12 °C	16 °C	12 °C	16 °C	12 °C	16 °C	12 °C	16 °C
CO <sub>2</sub> level	380 ppm	380 ppm	750 ppm	750 ppm	380 ppm	380 ppm	750 ppm	750 ppm
NH <sub>4</sub> (μmol)	11.92	7.57	22.48	5.41	7.05	4.58	8.31	3.99
	$\pm 3.74$	$\pm 2.61$	$\pm 6.74$	$\pm 1.93$	$\pm 1.85$	$\pm 1.33$	$\pm 2.16$	$\pm 1.38$
PO <sub>4</sub> (μmol)	0.11	0.19	0.08	0.10	0.13	0.88	0.65	0.15
	$\pm 0.02$	$\pm 0.03$	$\pm 0.01$	$\pm 0.03$	$\pm 0.03$	$\pm 0.17$	$\pm 0.16$	$\pm 0.05$
Si (μmol)	0.77	1.08	0.58	1.51	1.12	1.77	1.66	0.87
	$\pm 0.43$	$\pm 0.44$	$\pm 0.16$	$\pm 0.84$	$\pm 0.38$	$\pm 0.37$	$\pm 0.39$	$\pm 0.24$
NO <sub>3</sub> + NO <sub>2</sub> (μmol)	18.58	40.11	21.84	27.70	1.14	10.21	5.38	0.70
	$\pm 4.04$	$\pm 6.54$	$\pm 3.77$	$\pm 7.33$	$\pm 0.50$	$\pm 3.84$	$\pm 2.93$	$\pm 0.18$
Dissolved Inorganic Carbon (mM)	2.63	2.79	2.71	2.83	2.33	2.42	2.42	2.43
	$\pm 0.04$	$\pm 0.06$	$\pm 0.04$	$\pm 0.05$	$\pm 0.02$	$\pm 0.02$	$\pm 0.02$	$\pm 0.02$
Total Alkalinity (mM)	2.83	2.97	2.88	2.88	2.58	2.67	2.65	2.65
	$\pm 0.05$	$\pm 0.05$	$\pm 0.06$	$\pm 0.07$	$\pm 0.02$	$\pm 0.03$	$\pm 0.02$	$\pm 0.03$





**Fig. 2.** (A) Meiofauna abundance for sediment-type-day groups of samples (average ( $\pm$  SD,  $n = 11$ ) in black and sample abundances in grey), (B) Box and whisker plot of meiofauna abundance (ind.  $10 \text{ cm}^{-2}$ ) for mud samples at day 28 with 380 ppmv ( $n = 6$ ) and 750 ppmv treatment ( $n = 5$ ). Box boundaries represent 25–75 percentile, line within box represents median, error bars indicate 90th and 10th percentiles. C–E) abundance (average ind.  $10 \text{ cm}^{-2}$ , error bars indicate 95% confidence intervals) comparison between 380 ( $n = 6$ ) and 750 ppmv ( $n = 5$ ) at 28 days in mud samples, C) nematodes, D) copepods, E) ostracods.

ence between  $\text{CO}_2$  treatments at day 0 ( $p = 0.039$ ) and not day 28 ( $p = 0.356$ ) in sandy sediments prior to exposure (pairwise comparisons in 3-way PERMANOVA (Ti, Te,  $\text{CO}_2$ ) in sandy sediments). The idea that time spent in the flume has an effect on meiofauna taxon richness was evidenced by the significant dispersion effects observed when comparing day 0 and day 28 in both sandy and muddy sediment groups (PERMDISP:  $p = 0.015$  and  $0.001$ , respectively), without finding significant temperature or  $\text{CO}_2$  effects.

Pielou's evenness was not a discriminative measure since none of the statistical tests revealed any significant differences. Some of the tests were hampered by the low number of taxa in some of the samples making the calculation of Pielou's index invalid, whilst other tests revealed no significant differences using the various PERMANOVA designs.

Four-way PERMANOVA testing on the Shannon diversity measure indicated a significant sediment-type difference across all time, temperature and  $\text{CO}_2$  exposure groups ( $p = 0.001$ ) which was not caused by dispersion differences (PERMDISP:  $p = 0.136$ ). This difference can be clearly observed in Fig. 3. No other main-factor significant differences in the various tests were observed (Table S2).

### 3.2.3. Meiofauna community structure

Four-way PERMANOVA testing indicated significant sediment-type differences across all groups ( $p = 0.001$ ) and several significant interactions (Table S1). Sediment type differences were caused in part by differences in dispersion with clearly greater community variability in the mud samples compared to a closer resemblance between sand samples (PERMDISP:  $p = 0.006$ ; nMDS in Fig. 4). Pairwise testing for the significant Time  $\times$  Sediment interaction revealed a borderline difference between day 0 and day 28 for mud samples ( $p = 0.06$ ) and

slightly greater community differences between sand and mud samples at day 28 ( $p = 0.004$ ) compared to day 0 ( $p = 0.004$ ). Pairwise comparisons within the Time  $\times$   $\text{CO}_2$  interaction indicated a significant difference between 380 ppmv and 750 ppmv at day 0, suggesting the difference resulted from differences between the starting communities and not the different  $\text{CO}_2$  exposure. Pairwise tests showed that the significant Sediment  $\times$  Temperature interaction was caused by significant differences between sediment types at  $12^\circ\text{C}$  and  $16^\circ\text{C}$  ( $p = 0.001$  for both) and not the community differences between  $12^\circ\text{C}$  and  $16^\circ\text{C}$  in each sediment type ( $p = 0.25$  and  $p = 0.238$ ). The significant Time  $\times$  Sediment  $\times$  Temperature interaction was caused by sediment-type differences and greater community variability in the  $16^\circ\text{C}$  group for both sand and mud samples ( $p = 0.015$  and  $0.05$ , respectively). Separating the mud and sand data and performing 3-way PERMANOVA tests did not add anything new to these interpretations, apart from confirming a difference in day 0 communities (for instance between 380 ppmv and 750 ppmv in sandy sediments,  $p = 0.03$ , Table S1). Taking the sediment-type-time data as separate sets, and conducting 2-way PERMANOVA tests, did not add anything new to what is reported above.

### 3.3. Nematode diversity and community structure

#### 3.3.1. Nematode diversity

A total of 39 nematode genera were identified, averaging  $12.2 \pm 4.5$  genera per sample, with genus richness ranging between 4 and 19 in samples. Nematode genus richness differed significantly between sediment types in the 4-way PERMANOVA test ( $p = 0.001$ , Table S2, also see Figs. 5, 6). When splitting the data into mud and

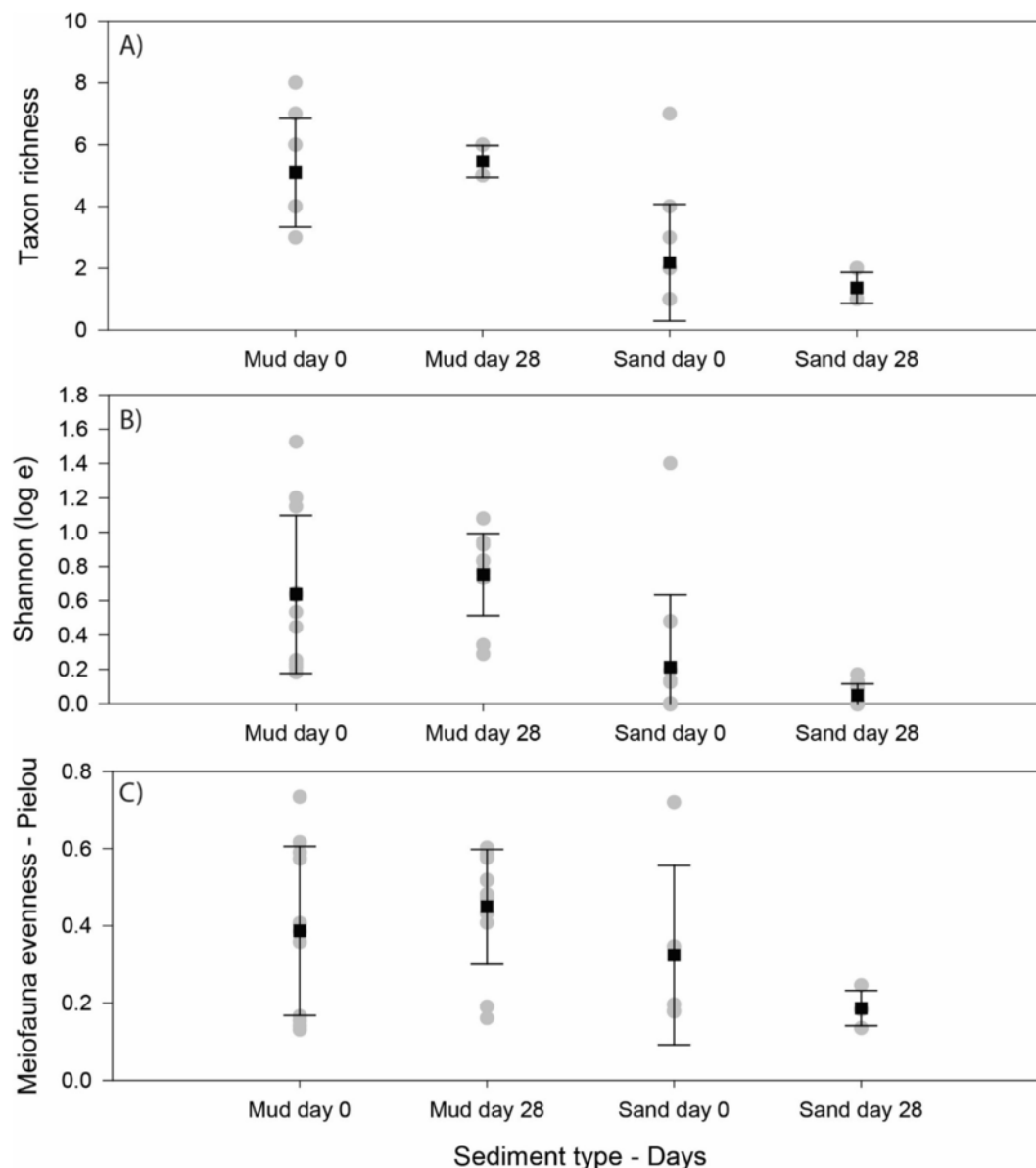


Fig. 3. Meiofauna diversity measures for sediment-type-day groups of samples (average ( $\pm$  SD,  $n = 11$ ) in black and sample abundances in grey). (A) Meiofauna taxon richness (S), (B) Meiofauna Shannon diversity (log  $e$ ), (C) Meiofauna Pielou evenness.

sand groups, 3-way PERMANOVA tests revealed a time effect across the mud samples (day 0 vs. day 28,  $p = 0.037$ ). Further testing revealed a significant effect of CO<sub>2</sub> exposure level in the mud samples at day 28 (pairwise testing in 3-way PERMANOVA,  $p = 0.04$ ; 2-way test at day 28,  $p = 0.041$ ; Fig. 6). At day 28, mud samples exposed to 380 ppmv averaged  $14.2 \pm 2.3$  genera, compared to  $16.8 \pm 1.5$  genera in the 750 ppmv treatment (Fig. 6).

PERMANOVA testing on Pielou's evenness index ( $J'$ ) indicated significant differences between sediment types ( $p = 0.018$ ) and a CO<sub>2</sub> exposure effect after 28 days in the mud samples (2-way PERMANOVA test,  $p = 0.035$ , Table S2). The CO<sub>2</sub> exposure difference in evenness of mud samples at day 28 was more severe at 16 °C ( $p = 0.005$ ), whilst a temperature effect was observed in the 380 ppmv mud samples at day 28 ( $p = 0.024$ ).

PERMANOVA testing using Shannon Wiener index ( $H'$ ) showed a significant sediment-type effect ( $p = 0.004$ ) and an interaction effect between sediment type and CO<sub>2</sub> exposure level ( $p = 0.046$ ). Pairwise testing showed that the CO<sub>2</sub> exposure effect was only observed in the

mud samples ( $p = 0.034$ ). This observation was confirmed with the 3-way test on the mud sample group (CO<sub>2</sub> effect,  $p = 0.039$ ) and the 2-way test on mud samples at day 28 (CO<sub>2</sub> effect,  $p = 0.005$ ). In addition, mud samples exposed to 380 ppmv changed significantly in terms of Shannon diversity between day 0 and day 28 ( $p = 0.005$ ).

### 3.3.2. Nematode community structure

Whilst for the meiofauna community structure the mud samples showed greater heterogeneity compared to the sand samples, using nematode genera data the opposite pattern was observed (Fig. 7). The nMDS plots in Fig. 7 with superimposed SIMPROF analyses presents clearly the greater variability in nematode communities in sand samples compared to the mud samples.

The 4-way PERMANOVA test on the nematode community indicated a clear sediment-type and time effect ( $p = 0.001$  and  $0.044$ , respectively). Fig. 8 shows the relative abundance of genera (10 most important genera in terms of relative abundance) and gives an idea of the genus composition in sand and mud samples, with mainly *Metachro-*

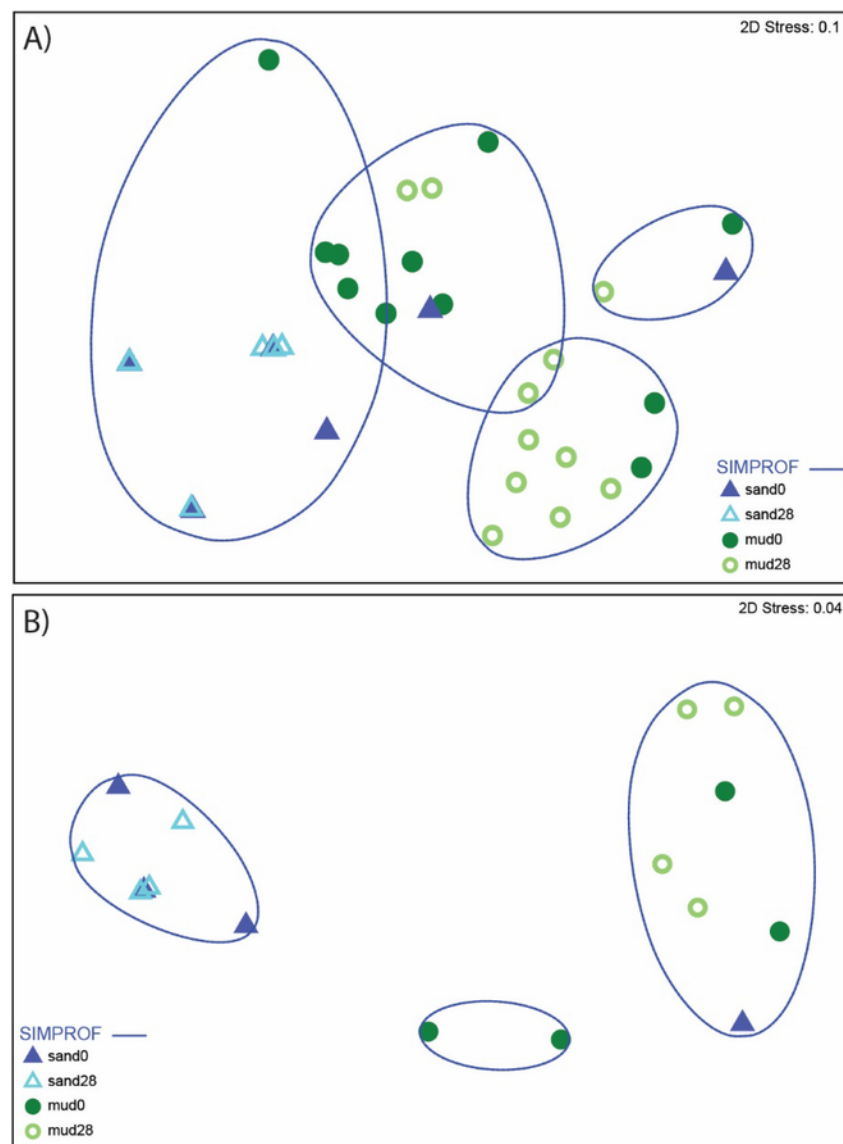


Fig. 4. Non-metric MDS plots on meiofauna major taxa abundance (standardised, 4th root transformation, Bray Curtis resemblance). Symbols represent samples belonging to two sediment type (sand, mud) and two time (0 days, 28 days) combinations. A) all samples, B) samples averaged over replicates. Lines indicate SIMPROF significance at the 5% level.

*madora* dominating the sand samples and *Anoplostoma*, *Metachromadora* and *Ptycholaimellus* dominating the mud samples. PERMDISP analysis indicated that the differences between sediment types is in part caused by the differences in dispersion of mud vs. sand samples in Bray-Curtis space ( $p = 0.001$ ), i.e. communities were more heterogeneous in the sand samples compared to the mud samples. The time effect was consolidated in the mud sample data (3-way PERMANOVA, Time:  $p = 0.022$ ). There were no signs of a temperature or  $\text{CO}_2$  exposure effect. This suggests that time spent in the flume may have had an effect on the nematode communities, particularly in the mud sediments, regardless of the different treatment levels.

### 3.4. Experimental controls – Campaign (sampling time) and flume effects

For each of the meiofauna and nematode abundance, diversity and community data sets, 2-way PERMANOVA tests were carried out to assess the effects of campaign (time at which the samples were taken before transfer to the flume) and the flumes (six flume tanks were used). Results of these tests can be found in Table S3 (meiofauna) and Table S4 (nematodes).

For the meiofauna data, very few significant differences for the main factors were observed. For meiofauna Pielou evenness, there was a significant effect of Campaign and Flume ( $p = 0.001$ ,  $0.005$ , respectively) which was not caused by differences in homogeneity of dispersions (PERMDISP,  $p = 0.824$ ,  $0.713$  for factors Campaign and Flume, respectively). The fact that no treatment effects on meiofauna Pielou evenness were found (see above), suggests that only effects caused by differences in flume setup and/or the time of sampling (Campaign) were present. Subtle Campaign effects were observed on meiofauna Shannon diversity and community structure for mud samples on day 28 (Table S3) suggesting the time of sampling before starting the experiment may have interfered with the treatment effects observed after 28 days of experiment as presented in section 4.1.1–4.1.3.

For the nematode full data set (both sand and mud samples together), clear Campaign and Flume effects were observed (Table S4) for all diversity descriptors (genus richness (S), Pielou's evenness, Shannon diversity), but only Flume effects for community structure. Further Flume effects were also observed on nematode genus richness and Shannon diversity, when only sand samples were considered, whilst Campaign effects were significant for Pielou evenness in sand samples



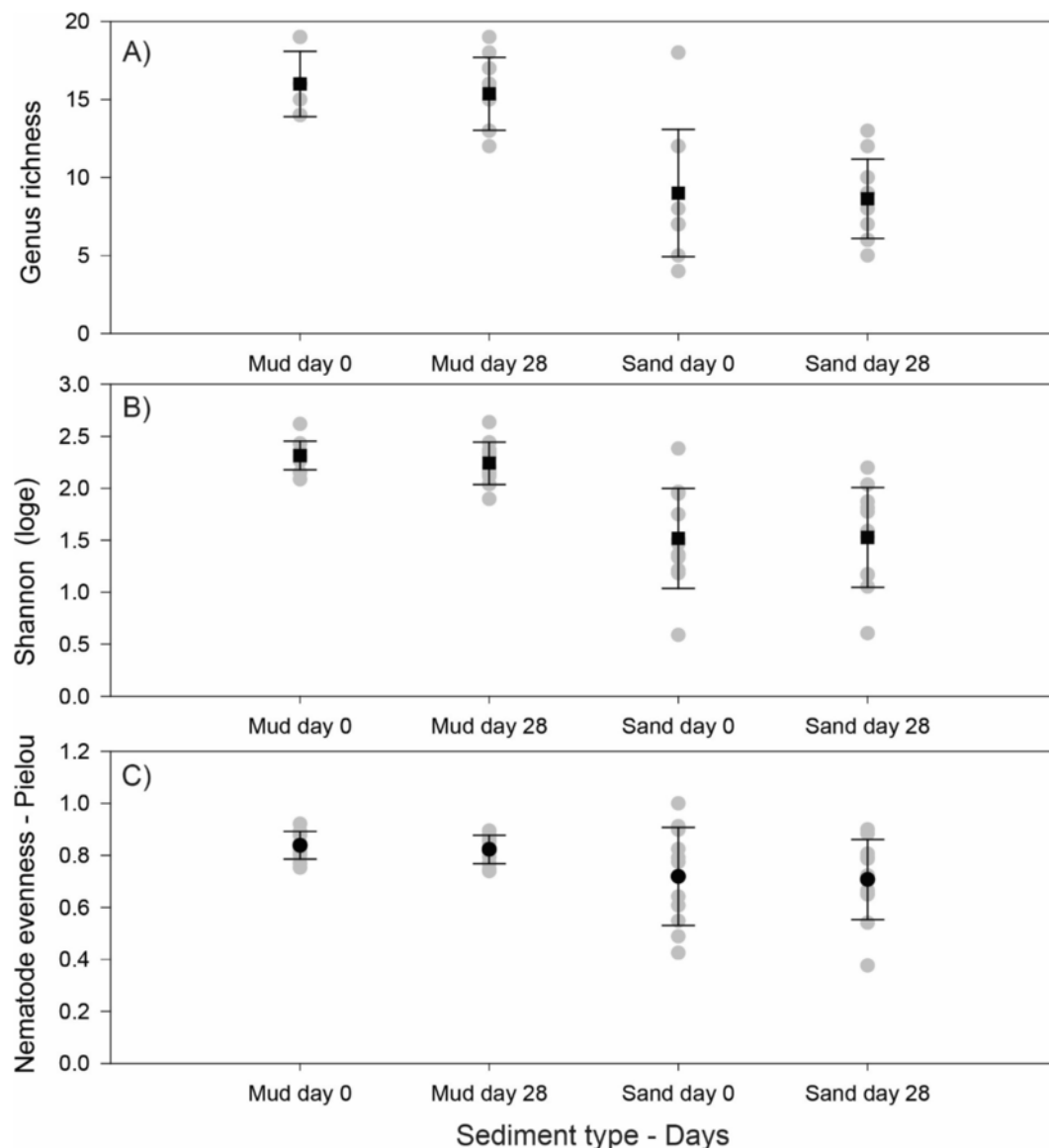
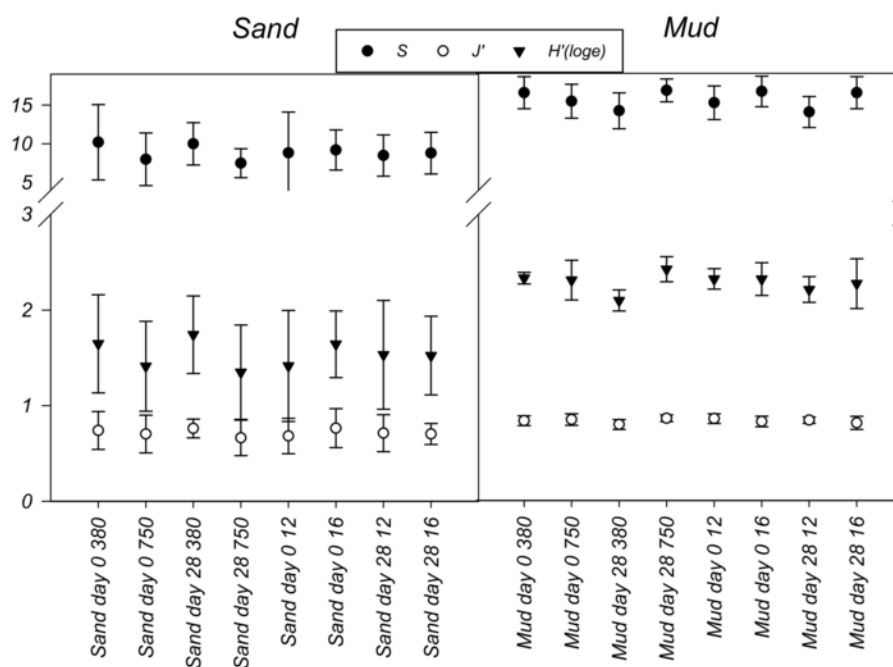


Fig. 5. Nematode diversity measures for sediment-type-day groups of samples (average ( $\pm$  SD,  $n = 11$ ) in black and sample abundances in grey). (A) Nematode genus richness (S), (B) Nematode Shannon diversity (log e), (C) Nematode Pielou evenness.

and sand samples at day 0, and for community structure in mud samples and mud samples at day 0. This suggests the time of sampling prior to the experiment (Campaign) and the different flumes may have had an additive effect to the treatment effects observed (Time, CO<sub>2</sub>, Temperature). Notably the significant interaction terms (Ca  $\times$  Fl) and pairwise differences suggest that there were differences between particular pairs of campaigns, flumes and levels of one factor (campaign or flume) within each level of the other factor (flume or campaign). For instance, the pairwise test for the factor Campaign on nematode genus richness using the full data set (mud and sand samples together) showed that Campaign 2 and 4 did not differ, and the same was true for Campaign 5 and 6, whilst pairwise comparisons between Campaigns of these two groups (i.e. 2 vs. 5, 2 vs. 6, 4 vs. 5, 4 vs. 6) were significant at the  $p < 0.05$  level. Pairwise comparisons for the factor Flume on nematode genus richness (again using mud and sand sample data together) indicated that Flume 1 was significantly different from all other Flumes. When we performed pairwise comparisons for nematode Pielou evenness we observed that Campaign 5 was different to all others, and that Flume 1, again, was different from all other Flumes.

For Shannon diversity, Campaign 6 was different to all other campaigns and Flume 1 stood out in contrast to the other Flumes. Finally, using nematode community structure, it was again Flume 1 that differed significantly, but this time only compared to some of the other Flumes.

This of course creates a complex picture of potential interactions between the effects caused by the experimental protocol (factors Campaign and Flume) and the treatment effects (Temperature, CO<sub>2</sub>), but also the duration of the experiment (i.e. day 0 vs. day 28). However, we can say with some confidence that for the meiofauna results, this will have made little or no difference in interpreting the treatment effects. For the nematodes, some caution is warranted since the effects of Campaign and Flume are more obvious, and this across the range of community descriptors. At the same time, however, such effects are only apparent when mud and sand samples are considered together, potentially indicating the importance of the differences between sand and mud samples. In addition, when investigating the differences between Campaigns and Flumes, it becomes clear that the differences are caused by a limited set and not the full set of samples within each



**Fig. 6.** Nematode diversity indices for each combination of sediment type, day and CO<sub>2</sub> exposure or Temperature, indicating consistent differences in nematode genus richness and Shannon Wiener diversity between sediment types, whilst Pielou evenness did not differ significantly and no differences were observed between CO<sub>2</sub> and temperature treatments ( $n = 5$  or  $n = 6$  depending on the treatment combination).

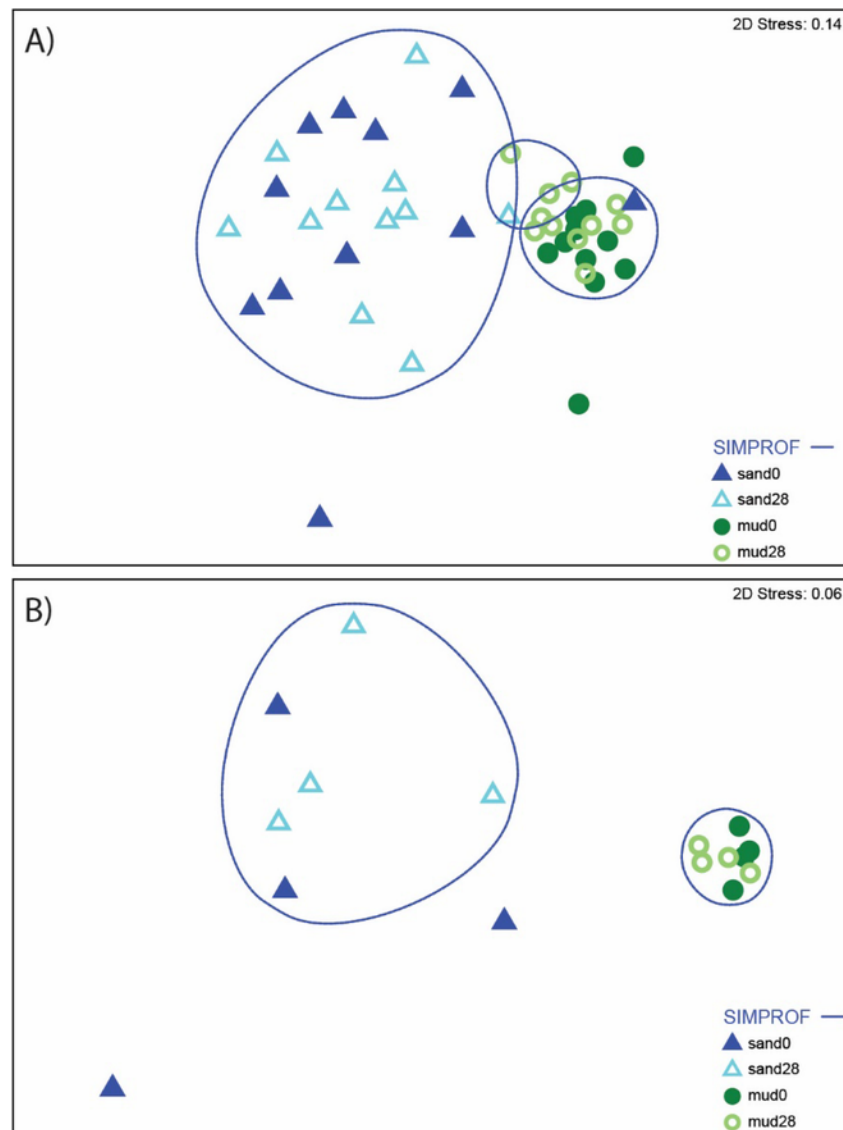
Campaign or Flume that differ significantly from the samples grouped within the other Campaigns or Flumes in what is in essence a type of outlier effect on the overall result. This implies that Campaign and Flume effects are not uniform across all the samples considered, and reduces the potential additive influence of Campaign and Flume effects on the Temperature and CO<sub>2</sub> effects.

#### 4. Discussion

Ocean acidification and warming are expected to have a range of impacts on marine species, populations and communities (Pörtner, 2008; Pörtner et al., 2004; Widdicombe, 2009; Widdicombe et al., 2011). Whilst the majority of meiofauna and nematode OAW studies have detected limited impacts of changes that lie within the expectations of global warming within the next century or so, there are well-founded concerns about the potential impacts on organismal physiology and energy allocation under increased levels of stressors (Pörtner, 2008; Pörtner and Farrell, 2008). Here we report on the community level responses of meiofauna and nematodes to a 4 °C increase (12 vs. 16 °C) and increased CO<sub>2</sub> concentration (380 vs. 750 ppmv). Our interpretations are therefore limited to assessing differences and variability on the community level, which give insights into the trade-off between species or groups of species, and potentially the underlying causes of community changes. The sediments used in our experiment were sieved to exclude macrofauna, and our experimental setup precludes any influence from pelagic organisms, such as phytoplankton communities. It could be that the biggest impacts in meiofauna from OAW is or will be from changes in the communities with which they interact strongly; e.g. macrofauna (predation, competition for food, etc.) or phytoplankton (e.g. changes to the type and quality of organic matter). We therefore note that our study focuses more on the direct impact of OAW on the meiofauna themselves, and the indirect impacts resulting from interactions with microbial and microphytobenthos communities.

##### 4.1. Meiofauna

Without doubt the most important driver for the abundance, diversity and community differences we observed was sediment type, and this both at the beginning (day 0) and the end (day 28) of the experiment, and across the different temperature and CO<sub>2</sub> treatments. Muddy sediments were characterised by much higher meiofauna abundances than the sandy sediments (Fig. 2), an observation that can potentially be related to the higher density of microbial food sources in muddy sediments compared to sandy sediments (Currie et al., 2017). Higher respiration rates in the muddy sediments as indicated by DIC results are supported by higher meiofauna abundance in those sediments and the higher abundance of bacterial, archaeal, and cyanobacterial 16S rRNA genes in muddy sediments compared to sandy sediments (Currie et al., 2017). Sediment type and immediately related parameters such as grain size, surface area, porosity and permeability are key factors since they determine the physical and chemical environment of the interstitial space inhabited by the meiofauna and affects food availability for the meiofauna. Whilst mesobenthic species moving between the sand grains prefer coarse sands, *endo*- and *epibenthic* species will generally prefer fine to silty sediments (Giere, 2009). It is therefore not surprising that meiofauna tend to be more sensitive to changes in sediment composition than the macrofauna (Heip et al., 1985; Warwick and Buchanan, 1970), which makes them a useful faunal component to detect benthic environmental change in an OAW experiment using different sediment types. The 11-year meiobenthos study by Coull (1985) in which he investigated subtidal estuarine muddy and sandy sites in South Carolina, USA, showed that variability in meiofauna abundance at the mud site was twice that of the sand site they studied. This corroborates our observation that meiofauna abundance in the mud samples varies much more compared to abundance in the sand samples (Fig. 2). This pattern was also visible in the meiofauna community variability as shown in the nMDS of Fig. 4, with greater dispersion of the mud samples compared to the sand samples in Bray Curtis resemblance space. However, considering the seasonal and interannual abun-



**Fig. 7.** Non-metric MDS on nematode genera abundance (standardised, sq. root transformation, Bray Curtis resemblance). Symbols represent samples belonging to two sediment type (sand, mud) and two time (0 days, 28 days) combinations. (A) all samples, (B) samples averaged over replicates. Lines indicate SIMPROF significance at the 5% level.

dance variability Coull (1985) had found and as reported in the review by Giere (2009), we were surprised to see no campaign effect (i.e. seasonal) on meiofauna abundance, particularly in the mud samples where highest and consistent seasonal variability was expected (Table S3). Despite abundance peaks of over 5000 ind.  $10\text{ cm}^{-2}$  in mud samples of Campaign 4 (April) and peaks of over 7000 ind.  $10\text{ cm}^{-2}$  in mud samples of Campaign 5 (June), the large differences between mud sample abundances resulted in a lack of significant Campaign differences and may be related to spatial variability of meiofauna across the samples. For instance, meiofauna and nematodes are known to have aggregated distribution patterns in virtually all marine habitats with patch sizes smaller than 5 cm in diameter, mainly in response to microtopographic irregularities and aggregate distribution of food sources (Moens et al., 2013 and references therein). It therefore meets expectations that the spatial scale of cm to m is generally found to be the most important source of variability for meiofauna organisms (Fonseca et al., 2010; Ingels and Vanreusel, 2013; Moens et al., 2013; Rosli et al., 2016; Vieira and Fonseca, 2013).

Aside from the sediment effect, there was also a significant  $\text{CO}_2$  exposure effect on meiofauna abundance in the mud samples at day 28,

which is visualised in the box-whisker plot in Fig. 2. This suggests a moderately negative impact on meiofauna abundance in muddy sediments. When investigating each major taxon, it became apparent that the density decrease was caused by a reduction across most taxa, with major decreases in numbers of nematodes, copepods, and ostracods (Fig. 2). This pattern is in line with our expectations based on the likely metabolic cost of coping with high  $\text{CO}_2$  concentration, and potentially the resulting reduction in fitness and survival. Some studies have shown that the meiofauna community may exhibit significant mortality following  $\text{CO}_2$  exposure; e.g. decreased meiofauna abundance associated with a rapid pH drop of  $\sim 1.5$  in deep-sea sediments in Carman et al. (2004) and Barry et al. (2004) or meiofauna abundance decrease under increased  $\text{CO}_2$  concentration conditions of 20,000 ppmv at 2000 m water depth in the Kumano trough off Japan (Ishida et al., 2005). Ishida et al. (2013), on the other hand, reported no abundance decline under increased  $\text{CO}_2$  exposure at 400 m depth in a Norwegian Fjord, but slow decomposition rates in deep cold waters (low microbial activity) may have caused an overestimation of meiofauna abundance in high- $\text{CO}_2$  treatments in the absence of specific staining to detect live/dead ratios (Carman et al., 2004). Explanations for abundance ef-

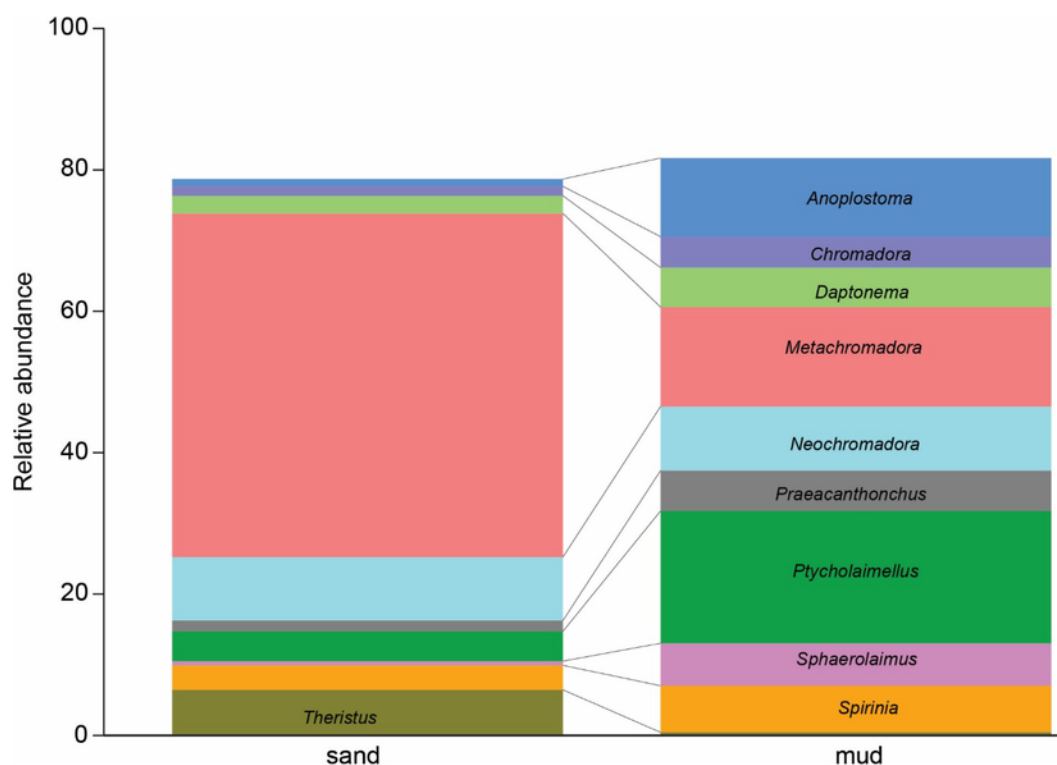


Fig. 8. Average relative abundance for the sand and mud samples representing only the 10 most important genera, indicating the dominance of *Metachromadora* in sand samples and the more evenly distributed community in mud samples.

fects other than mortality may include physiological processes and behaviour including escaping the unfavourable high- $\text{CO}_2$  conditions in the case of relatively mobile organisms such as copepods (Thistle et al., 2007). Observations of  $\text{CO}_2$  effects on meiobenthos in the deep sea, however, may not be comparable to shallow-water observations owing to the likely adaptation of deep-sea organisms to environmentally stable conditions and therefore increased sensitivity to environmental change compared to their shallow-water counterparts. Hale et al. (2011) and Meadows et al. (2015), for instance, reported unaffected or even increased nematode abundance under high- $\text{CO}_2$  conditions, likely as a result of reduced macrofaunal competition and predation, whilst Schade et al. (2016) and Kurihara et al. (2007) found no nematode and meiofauna abundance response to high  $\text{CO}_2$  treatments (1500–24,400  $\mu\text{atm CO}_2$  (Schade et al., 2016); 2000 ppmv above the 380 ppmv  $\text{CO}_2$  control level (Kurihara et al., 2007)). At the same time, Schade et al. (2016) reported that the abundance of non-dominant, calcifying meiofauna (gastropods and ostracods) declined in response to high  $\text{CO}_2$  concentration, whilst the non-calcifying gastrotrichs increased in abundance in response to very high levels of  $\text{CO}_2$  concentration. Meadows et al. (2015) reported that other meiofauna groups such as copepods, copepodites and amphipods decreased in abundance in low-pH treatments, suggesting that different meiofauna taxa and even different species within the same group (e.g. nematodes in Takeuchi et al., 1997; copepods in Thistle et al., 2006) have different tolerances to  $\text{CO}_2$  exposure. It is important to note here that one should distinguish between pH reductions or  $\text{CO}_2$  concentrations that can be associated with ocean acidification and those associated with the simulation of point-source leakage in the context of Carbon Capture and Storage (CCS). Most, if not all, studies investigating  $\text{CO}_2$  impacts on meiofauna abundance report significant effects only when applying severe pH reductions in experimental setups (e.g. > 1 pH unit change, or even an incredible pH 5.5–6 in Takeuchi et al. (1997)) and are not comparable to pH reductions of 0.1 or 0.2 units as is the case here. Reductions in pH of > 1 unit are not associated with ocean acidification as predicted under

climate change scenarios, and suggest that meiofauna in general are not affected in terms of abundance as a result of short- to long-term exposure to ocean acidification. This is another reason why deep-sea studies are difficult to compare to shallow-water studies; so far deep-sea studies have mainly addressed the impacts of potential  $\text{CO}_2$  storage or leakage on benthic assemblages in CCS contexts instead of looking at ocean acidification impacts.

Going back to the results of the present study, we need to consider the fact that the sediments were sieved to remove macrofauna before incubation in the flume system. A decrease in meiofauna abundance as a result of  $\text{CO}_2$  exposure is therefore likely a direct effect and not an indirect macrofauna effect caused through reduced interactions with potentially more severely affected macrofauna (Dashfield et al., 2008). Indirect effects, however, may have resulted through altered meiofauna-microbial interactions and may play an important role in our results. Microbial data from our experiment (Currie et al., 2017) indicate significant negative  $\text{CO}_2$  effects on bacterial, archaeal and cyanobacterial abundance (as assessed through 16S rRNA gene abundance) in muddy sediments after 28 days in the experiment. Considering microbiota are important food sources for meiofauna (e.g. Montagna, 1984) and particularly nematodes it is likely that a reduction in available food may have had consequences for meiofauna abundance. We also have to consider the fact that a negative  $\text{CO}_2$  effect on meiofauna abundance was only observed in the muddy sediments (and not the sandy sediments) which corresponds with the substantial microbial abundance decline observed only in muddy sediments (Currie et al., 2017). This supports the likelihood of an indirect  $\text{CO}_2$  effect on meiofauna abundance through a microbe-meiofauna relation, most likely a trophic interaction. In addition, we have to consider a potential indirect trophic effect on the meiofauna through a change in abundance and community structure of the microphytobenthos which are prevalent in intertidal muddy sediments. In the same experiment cyanobacterial/chloroplast 16S rRNA gene abundances were reduced and microbial community structure was altered under increased  $\text{CO}_2$  in the muddy sediments,

yet, gene abundance increased in the higher temperature treatment alone (Currie et al., 2017). Microphytobenthos is a well-known food source for different meiofauna (Lebreton et al., 2012; Montagna et al., 1995; Rzeznik-Orignac et al., 2008) in intertidal and other coastal marine systems and observations from a previous experiment indicate that there may be a tight correlation between meiofauna and microphytobenthos responses to OAW (Unpublished meiofauna data from another experiment, (Tait et al., 2015)). Trophic restructuring of the meiofauna community (and subsequent diversity changes) in response to a change in microphytobenthos abundance and community structure is a likely scenario since variation in trophic meiofauna types and their abundance has been closely linked with microphytobenthos consumption in intertidal systems (Montagna et al., 1995; Rzeznik-Orignac and Fichet, 2012). It is very likely that meiofauna abundance was reduced in response to reduced microphytobenthos availability as food source in the high CO<sub>2</sub> treatments. Alternatively, or additionally, the fact that a CO<sub>2</sub> effect occurred only in muddy sediments may be linked to different types of assemblages in the two sediment types and hence potentially different organism tolerance levels, carbonate chemistry, diffusion, and permeability variability between both sediment types.

In our experiment, cyanobacterial/chloroplast 16S rRNA gene abundance increased in the higher temperature treatment (Currie et al., 2017), yet no significant meiofauna response is observed for temperature alone. Considering meiofauna life cycles can be shortened under higher temperatures one may expect meiofauna abundance to increase in the high temperature treatment (Giere, 2009), particularly in the case of nematodes (Gerlach and Schrager, 1971; Heip et al., 1985; Hopper et al., 1973; Vranken et al., 1988; Warwick, 1981). However, the relatively short period (4 weeks) of our experiment may have been too short for such an effect to show given meiofauna reproductive cycles can vary between a couple of days and more than two months depending on the taxon and environmental conditions (Giere, 2009). In their experimental work on biofilm production and macrofaunal grazing during five weeks of OAW conditions, Russell et al. (2013) found that whilst primary production increased, consumer grazing decreased, suggesting increased energy expenditure of the consumer in response to their higher metabolic requirements under OAW. Such a scenario does not seem applicable to the meiofauna in our higher temperature treatments, suggesting that the intertidal meiofauna used here may experience relatively little additional metabolic cost under such conditions. Considering intertidal fauna experience extreme temperature gradients under natural conditions, our 4 °C temperature increase may have had little effect.

As mentioned at the beginning of this section, meiofauna taxon richness and Shannon diversity differed significantly between sand and mud samples, with consistently higher major taxon richness and Shannon diversity in muddy sediments compared to sandy sediments (Fig. 6). This supports the idea that sediment type and meiofauna diversity is linked as has been reported in several other studies (Giere, 2009 and references therein). The consistency of the sediment type differences across meiofauna diversity measures and across experimental treatments highlights the importance of sediment characteristics in determining the type of meiofauna communities that reside in them and the diversity they exhibit.

The CO<sub>2</sub> and temperature effects on meiofauna taxon richness for day 0 in sand and mud samples, respectively, and the CO<sub>2</sub> effect on meiofauna community structure in day 0 sand samples (Table S1) may be caused by differences between the starting communities or pre-treatment of the sediments (irrespective of sediment type) and does not represent a true CO<sub>2</sub> or temperature effect as a result of the different experimental treatments. Indeed, these differences occur at day 0, before exposure was initiated. Such initial distinction between assemblages is likely representative of assemblage differences as observed in the field, and potentially relates to differences associated with the

month in which the samples were taken. Results from the Campaign x Flume statistical tests on meiofauna confirm this to some extent, with a significant Campaign effect on Pielou evenness (sand samples), and Shannon diversity and community structure for day 28 mud samples (Table S3), implying that initial diversity and community differences may persist to some extent through 28 days of experimental exposure. Further evidence for high initial variation can be provided by comparing the standard deviations of the respective diversity measures between day 0 and day 28 samples for each sediment type (each sediment type taken separately to remove the overwhelming sediment type effect): a clear pattern of “shrinking variation” of the meiofauna diversity measures over the duration of the experiment is apparent. This is illustrated by the decreasing size of standard deviation bars of the diversity values over time in Fig. 3. We have presented this pattern more clearly by plotting the size of the standard deviations of each meiofauna diversity measure in Fig. 9, as it clearly shows that variation decreases as communities and assemblages are exposed to 28 days of experimental conditions. It is remarkable that, despite the differences in absolute values of diversity measures between sandy and muddy sediments (cf. size of the bars in Fig. 3), the variation – as judged by standard deviation – in these values is in fact very similar. This suggests that the observed “shrinking variation” occurs irrespective of the sediment type, and likely irrespective of the season or month in which the samples were taken from the field.

#### 4.2. Nematodes

As was the case for the meiofauna, the most pronounced difference for nematode diversity and community structure was caused by sediment type. This is clearly shown in Figs. 5–8 (arguably most clearly in the separation between sand and mud samples in the nMDS in Fig. 7). Although nematode density tends to increase in finer sediments such as mud and fine silt, diversity has been reported to be higher in coarser sediments (Heip et al., 1985; Steyaert et al., 1999). This pattern has been ascribed to increased microhabitat heterogeneity thought to be available in the latter (Heip et al., 1985) which could support more diverse communities with more closely related species co-existing (Steyaert et al., 1999). In sediments finer than about 120 µm a true interstitial fauna is lacking and a poorer burrowing fauna remains. However, such reasoning omits the potential of meiofauna and in particular nematodes to manipulate the fine sediments by means of their own

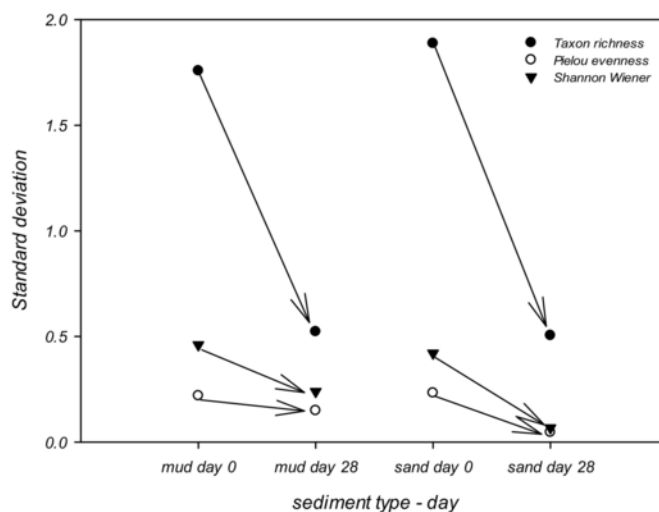


Fig. 9. Standard deviations of meiofauna diversity measures for the two sediment types at day 0 and day 28, indicating a consistent decline of variation over the duration of the experiment.

bioturbation activities and causing increased microhabitat heterogeneity as well as the stimulation of biogeochemical processes (Bonaglia et al., 2014). In addition, fine sediments are usually associated with greater concentrations of nutrients contained between the fine grains and may promote coexistence of nematode genera and species. Moreover, the manipulation of the sediments prior to our experiment by means of sieving has excluded the presence of macrofauna and hence their contribution to microhabitat heterogeneity. Our results clearly suggest a higher nematode diversity in muddy sediments compared to sandy sediments, in favour of the idea that higher food availability in muddy sediments allows for more diverse nematode assemblages. This is supported by the enhanced levels of nutrients that were found in muddy compared to sandy sediments in our experiment. Despite muddy sediments being more diverse in our study, community variability was much greater in sandy sediments compared to muddy sediments (Fig. 7). The main reason for this may well be related to the much lower numbers of organisms recovered from the sand samples compared to the mud samples. The Bray-Curtis similarity measure we used is very sensitive when comparing low-abundance samples (e.g. comparing samples with one individual each can result in 100% or 0% similarity), and disparity between samples with low numbers of individuals may therefore become inflated compared to samples with higher abundance (Clarke and Gorley, 2015). Regardless of what caused the greater variability of nematode communities in sandy sediments compared to muddy sediments, it is clear that they are indeed very different. The nMDS plots in Fig. 7 show a high-level distinction between nematode communities from both sediment types. Sediment characteristics such as organic matter content, porosity, permeability, grain size, etc. are key factors in determining the community that is present in different sediment types. We will not go into much detail on which genera were present in sandy versus muddy sediments in our experimental samples, but our data corresponds with findings of several other studies and support the distinction between sediment types based on nematode communities (e.g. Somerfield and Warwick, 1996). For instance, chromadorid genera (particularly *Metachromadora*) dominate sandy sediments much more than muddy sediments (Fig. 8), and typical genera such as *Anoplostoma* and *Ptycholaimellus* are prevalent in the muddy sediments (e.g. Heip et al., 1985; Moens et al., 2013; Netto and Gallucci, 2003; Yodnarasri et al., 2008 to name but a few). Our results also support the notion that nematodes appear very sensitive to even slight changes in sediment composition (discussed extensively in Giere, 2009; Heip et al., 1985).

Focusing on temperature and CO<sub>2</sub> treatments, there are some significant differences that emerge from our statistical testing. A significant CO<sub>2</sub> effect is observed for nematode genus richness and Pielou evenness in mud samples of day 28 (higher values in 750 ppmv treatment), and for Shannon-Wiener diversity in mud samples and mud samples of day 28 (higher values in 750 ppmv treatment; Fig. 6, Table S2). So it appears that the increased CO<sub>2</sub> exposure used in our experiment is able to influence nematode diversity, but only in muddy sediments, and does so by increasing diversity. Despite the fact that it is generally acknowledged that nematodes are good indicators of any kind of disturbance or environmental change (Balsamo et al., 2012), we did not expect nematode diversity to be influenced - let alone increase - by CO<sub>2</sub> exposure. Meadows et al. (2015) reported no significant nematode diversity effect in their various pH treatments, nor did Schade et al. (2016) and Dashfield et al. (2008). Although nematode diversity effects have generally not been observed in high-CO<sub>2</sub> experiments to date, community differences in response to high CO<sub>2</sub> exposure have been reported (see Meadows et al., 2015, although Schade et al. (2016) reports no nematode community changes under high-CO<sub>2</sub> exposure). There are indications that different nematode species may respond differently to lower pH owing to different behaviour (activity, feeding behaviour), possession of different physiological needs and thresholds

(Takeuchi et al., 1997), as well as in response to predatory release from other predator nematodes or turbellarians if those predators were less tolerant to changing conditions. However, our experiment revealed no nematode community differences as a result of different CO<sub>2</sub> exposures. The only community structure effects we observed were caused by the differences between day 0 and day 28 (for sand samples and sand and mud samples together; Table S2).

Aside from some CO<sub>2</sub> effects, we also observed a temperature effect on nematode genus richness in mud samples. Meadows et al. (2015) found a consistent negative effect of higher temperature (16 °C vs. 12 °C) across pH treatments on average nematode species richness, expected number of species (ES[50]), and Pielou evenness, whilst we found the opposite: nematode genus richness was lower under higher temperature (also 16 °C vs. 12 °C) across both CO<sub>2</sub> concentrations in mud sediments (Fig. 10). One potential explanation is that opportunistic species (r-strategists) may have outcompeted multiple K-strategy species, having benefited from the higher temperature. That being said, there are no other temperature effects observed in any of the tests on the nematode data (e.g. evenness, community structure) suggesting it may be a straightforward loss of some genera in the community.

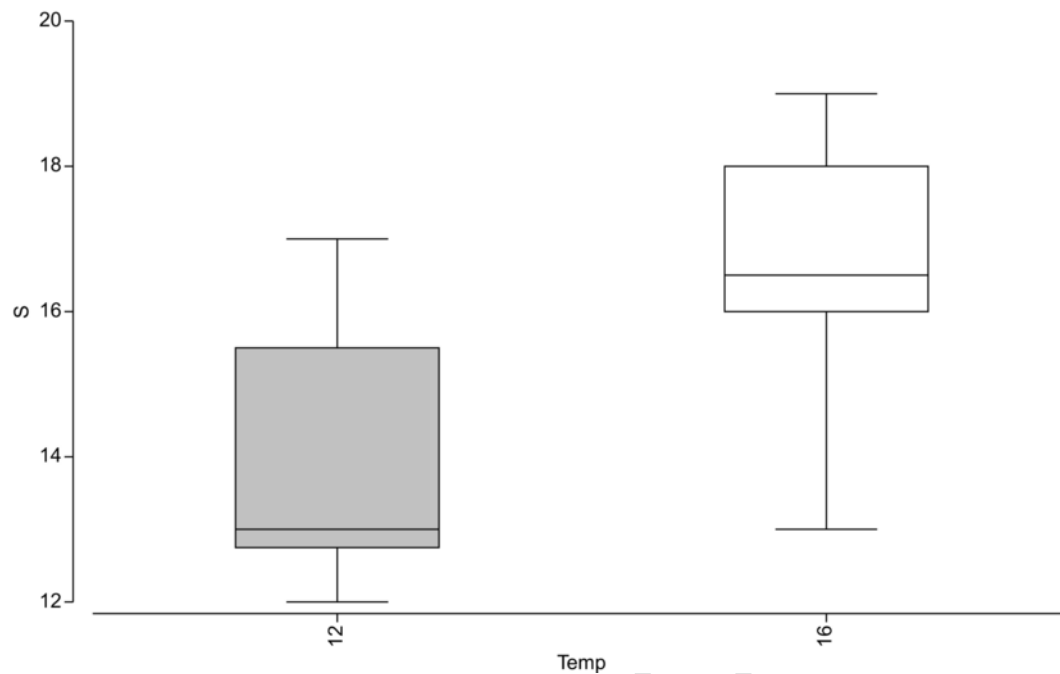
As for the meiofauna discussed above, nematode communities vary substantially on a monthly and yearly basis, with sediments sampled in different seasons exhibiting different abundance, diversity and community structure (Schratzberger et al., 2008; Vanaverbeke et al., 2004). Changes in nematode communities are best explained through food quality, quantity (availability) and temperature in sublittoral systems since these environmental characteristics may influence different species differently (reflecting different life histories and feeding modes), resulting in dynamic communities from season to season (Heip et al., 1985; Moens and Vincx, 2000; Schratzberger et al., 2008; Yodnarasri et al., 2008). The expectation that monthly and seasonal differences are likely to affect the nematode community characteristics (Yodnarasri et al., 2008) in our experimental samples was confirmed through the significant Campaign effects on nematode diversity values (mud and sand samples together; no significant result for community structure, Table S4). To investigate further the nature of the Campaign effects, we performed two-way tests on nematode genus richness and Shannon-Wiener diversity, using Campaign and sediment type as main crossed factors, and saw that sediment type effects remained significant whilst Campaign effects were borderline ( $p = 0.065-0.071$ ; no significant interaction term). This implies that the sediment effects were indeed distinguishable from potential Campaign effects, hence validating the sediment effects discussed above, but also that there are differences between the sampling months which were unlikely to influence the other effects observed (CO<sub>2</sub>, temperature, Time).

#### 4.3. Conclusions and future work

Our experiment has shown that the meiofauna and nematode communities originating from intertidal muddy and sandy sediments are relatively tolerant to the direct effects of OAW in a relatively short (4-week) mesocosm experiment. With regard to our hypotheses, we have shown that: (H1) OAW affects meiofauna and nematode communities to a very limited extent; (H2) meiofauna and nematode community responses to OAW are very different in muddy versus sandy sediments, with effects mostly found in muddy sediments; and (H3) Ocean acidification and warming together to do not present additive or synergistic effects on meiofauna and nematode community characteristics.

Sediment type was by far the most discriminating factor for meiofauna and nematode community structure, diversity, and evenness. There were negative CO<sub>2</sub> effects on meiofauna abundance in mud samples, caused by a reduction in abundance of notably the nematodes, copepods and ostracods. High CO<sub>2</sub> exposure resulted in increased nematode genus richness, Shannon diversity and Pielou evenness. The





**Fig. 10.** Box and whisker plots for nematode genus richness (number of genera, S) for both temperature treatments in mud sediments after 28 days. Box boundaries represent 25–75 percentile, line within box represents median, error bars indicate 90th and 10th percentiles ( $n = 22$  for both treatments).

only warming effect we observed, was a negative influence on nematode genus richness. In general, there were no significant interaction effects between CO<sub>2</sub> exposure and warming. These findings paint a complex picture whereby OAW influences meiofauna and nematode communities most likely through their food sources such as bacteria and microphytobenthos. Under OAW conditions, the ecological interactions may change: shifts in major taxa abundances and nematode genera trade-offs are likely consequences in benthic systems subjected to OAW.

Before meiofauna and in particular nematodes can be used as indicators for ocean acidification and warming a better understanding is needed of their spatial and temporal variation as well as improved knowledge on their physiology and life histories, and this in different environmental settings and marine habitats. Information on community responses do not necessarily provide a mechanistic view of the responses of the individuals and reasons behind different responses of species. As is often the case in meiofauna and nematode impact studies, information is often drawn from a multitude of studies in which particular meiofauna higher taxa or nematode genera or species have been observed to respond to a particular stressor or environmental change (Balsamo et al., 2012). In addition, our understanding of how different taxa and levels of biological organisation respond to ocean acidification and warming is limited, but it has been shown that different life stages will respond differently (Ingels et al., 2012; Kroeker et al., 2010). The ability to provide a comprehensive overview on meiofauna and nematode responses to ocean acidification and warming relies on studies which enable the separation of responses from higher taxa, species, and life stages to the different stressors. Moreover, and perhaps more importantly, insights into the ecological interactions between different meiofauna components as well as interactions between meiofauna and other biota are needed to achieve an understanding of true community responses to ocean acidification and warming. Our study has also shown that there is a need for integration between autecological and synecological studies and multi-stressor approaches to achieve an ecosystem view and to account for environmental interac-

tions and revealing additive or synergistic effects of different types of stressors (Zeppilli et al., 2015). It has previously been asserted that more multi-stressor experiments are needed to reveal complex ecological and biological interactions in a changing marine environment (Zeppilli et al., 2015). Finally, identifying meiofauna and nematodes is time-intensive and costly. Metagenomic barcoding may provide a more cost-effective way of identifying impact responses of communities. However, DNA-based meiofauna and nematode identification cannot yet rely on a species- and life-stage-specific understanding of the mechanistics of responses as well as the interactions and variability of these responses. It is therefore important that studies are conducted to provide mechanistic lower-taxon insights as well as community understanding of responses to OAW.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jembe.2017.07.012>.

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